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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This study aims to elucidate the signaling pathway of TRAIL-mediated apoptosis in prostate cancer cells, and to examine the therapeutic effect of TRAIL on prostate cancer cells in vitro and in vivo. We found that most of prostate cancer cells such as androgen-independent PC-3 and DU145 cells are sensitive to TRAIL treatment while normal prostate epithelial cells are resistant. This result indicates that TRAIL may be appropriate agent for treatment of late-stage prostate cancer with no cytotoxicity to normal prostate cells. Further investigation on the molecular mechanism of TRAIL resistance revealed that pro-survival protein kinase Akt existed in a constitutively active form at high level in LNCaP cells that are resistant to TRAIL. Inhibition of PI 3-kinase sensitized LNCaP cells to TRAIL, suggesting that Akt may play a critical regulatory role in TRAIL signaling. To further understand the working mechanism of Akt, we surveyed its downstream targets, and found that the elevated eNOS activity by Akt phosphorylation may partially contribute to Akt-mediated TRAIL resistance in LNCaP cells. Our preliminary result also indicated that other anti-apoptotic mechanisms may exist in LNCaP cells.

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INTRODUCTION:

Apoptosis or programmed cell death is a cell intrinsic suicidal mechanism, which is essential for tissue homeostasis and animal development. Dysregulation of this process may lead to a number of human diseases, including prostate cancer (PC). Resistance of PC cells to apoptosis plays important roles in the pathogenesis and progression of prostate cancer. Some of androgen-independent cancer cells that do not undergo apoptosis after androgen ablation become apoptosis-resistant and metastatic, frequently due to upregulation of anti-apoptotic Bcl-2 and loss-of-function of p53 (Bookstein et al, 1993; Eastham et al, 1995; Cardillo et al, 1997). As a result, metastatic prostatic cancer, usually resistant to conventional anti-tumor therapies, is a lethal disease without curative therapy. Therefore, an understanding of apoptotic machinery and its regulatory mechanism in PC cells is critical for us to develop an effective therapy to combat metastatic prostate cancer. The TNF-related apoptosis-inducing ligand, TRAIL (also called Apo2L), has recently been emerging as a non-toxic anti-cancer agent because it is capable of inducing apoptosis in many of tumor cell lines but not in normal cells tested (Wiley et al, 1995; Pitti et al, 1996). In this project, we will examine the effects of TRAIL treatment in androgen-dependent and -independent PC cells, to dissect the TRAIL-induced signaling pathway in those cell lines, and to characterize the synergistic effects of TRAIL with other therapeutic agents, and finally to test the anti-tumor effect of TRAIL in the animal models of prostate cancer. Through these works, we will be able to firmly establish the foundation for a novel therapy of prostate cancer.

BODY:

Task 1: To examine the TRAIL-induced apoptotic pathway in prostate cancer cells (months 1-12):

a. To survey TRAIL sensitivity of PC cells (months 1-6)

Results:

Most of prostate cancer cells are sensitive to TRAIL:

We tested the sensitivity of five prostate cancer cell lines and normal prostate epithelial cell to purified TRAIL. As shown in Figure 1A, except LNCaP, most of prostate cancer cells were sensitive to TRAIL to different extents. Among them, androgen-independent cells such as PC-3, DU145 that represent the later metastatic prostate cancers were most sensitive to TRAIL, indicating that TRAIL may be effective for treatment of metastatic prostate cancers. Additionally, normal epithelial prostate cancer cells were resistant to high concentration of TRAIL, further confirming the previous observation that TRAIL-induced apoptosis is specific for tumor cells.

A. B.

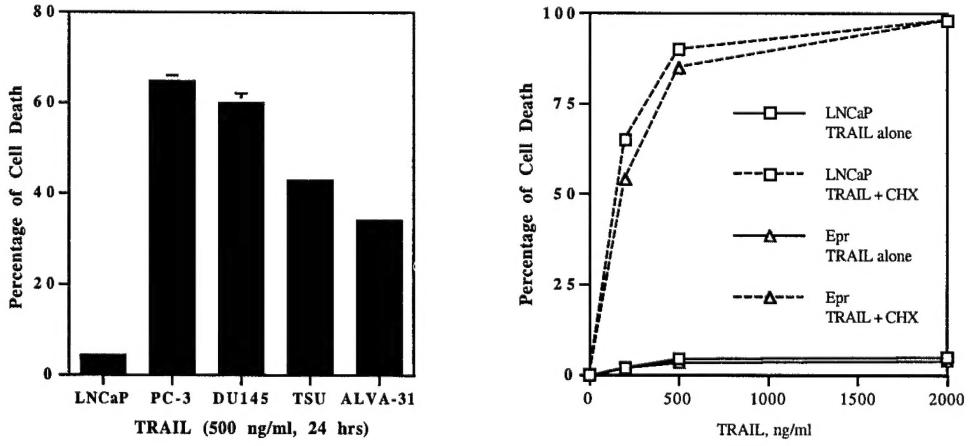

Figure 1. TRAIL sensitivity of normal prostate epithelial cells and various prostate cancer cells. A. TRAIL sensitivity of 5 PC cells. PC cells were treated with purified His-tagged TRAIL for 24 hours, and the percentage of cell death was measured with MTT assay. B. The effect of cycloheximide (CHX)

on TRAIL-induced apoptosis of LNCaP and prostate epithelial cells. Cells were treated with various amount of TRAIL in the absence or presence of cycloheximide (10 μg/ml) for 24 hours, and the percentage of cell death was evaluated by MTT assay.

b. To examine the effect of cycloheximide on TRAIL sensitivity of prostate cancer cells (months 6-9)
Results:

Inhibition of protein synthesis sensitizes PC cells to TRAIL

As shown in Figure 1B, inhibition of protein synthesis by cycloheximide sensitized LNCaP and normal prostate epithelial cells to TRAIL. Two conclusions can be drawn from this results: 1) the protein apparatus mediating TRAIL signaling, including TRAIL receptors, adaptor proteins and caspases, is intact in LNCaP and normal cells since the combination of TRAIL and CHX efficiently induces these cells to undergo apoptosis; and 2) a short-lived inhibitor of apoptosis may block TRAIL signaling in LNCaP and normal cells.

c. To identify the possible molecular determinants of TRAIL sensitivity (month 6-12)
Results:

Despite the fact that TRAIL signaling pathway remains poorly understood, it is generally believed that TRAIL signaling is similar to Fas signaling pathway, in which cross-linking of death receptors leads to the formation of Death-Inducing-Signaling-Complex (DISC) and activation of caspase-8. Active caspase-8 activates downstream caspase cascade and induces mitochondrial damage via BID cleavage, and results in apoptotic cell death. However, TRAIL signaling seems to be regulated by multiple factors at multiple levels. To identify the possible molecular determinants of TRAIL sensitivity, we have screened the known regulators for TRAIL signaling pathway.

1) The apoptotic machinery in LNCaP is intact.

As indicated in the experiment of cycloheximide, the TRAIL signaling pathway in LNCaP cells seems to be intact even though cells are fully resistant to TRAIL. We examined the components of TRAIL signaling pathway in LNCaP and other TRAIL sensitive cells, including TRAIL receptors DR4 and 5 (Figure 2A), adapter protein FADD, and caspase 3, 7, 8 (data not shown). We found no correlation between the sensitivity of the cells to TRAIL and the expression of various components. All cells express the apoptotic components we have examined. Additionally, we examined some of apoptotic inhibitors such as Bcl-XL and XIAP that may contribute to TRAIL resistance, and no correlation was found (Figure 2A).

2) LNCaP cells contain high level of active Akt

LNCaP cells have been shown to contain no tumor suppressor gene PTEN (Vlietstra et al, 1998), a protein and lipid phosphatase that negatively regulates PI 3-kinase signaling. PI 3,4,5-triphosphate produced by PI 3-kinase activates protein kinase Akt that further inactivates pro-apoptotic factors such as BAD and caspase-9 (del Peso et al 1997; Datta et al 1997; Cardone et al 1998), which in turn provides a survival signal. As shown in Figure 2B, only LNCaP cells contained high level of constitutively active Akt even though all cell lines expressed similar level of Akt. Furthermore, wortmannin (100 nM), an inhibitor of PI-3 kinase, sensitized LNCaP cells to TRAIL (Figure 2C). This result strongly suggested that constitutively active Akt may contribute to TRAIL resistance of LNCaP cells. Similar observation has been reported by Nesterov et al (2001) and Thakkar et al (2001).

3) eNOS (endothelial Nitric Oxide Synthase), an Akt substrate, is anti-apoptotic in PC cells.

To further investigate the role of Akt in TRAIL signaling, we examined expression and phosphorylation of the known Akt substrates. While phosphorylation of BAD and caspase-9 did not correlate with TRAIL sensitivity among PC cells (data not shown), endothelial Nitric oxide synthase (eNOS), another Akt substrate, was found only in LNCaP cells (Figure 2B). Previous studies have shown that phosphorylation of eNOS at residue ser1179 by Akt can enhance its enzymatic activity (Fulton et al, 1999; Dimmeler et al, 1999). Moreover, nitric oxide has been shown to act as an apoptotic

inhibitor in certain types of cells via nitrosylation of caspases (Mannick et al 1999). We hypothesized that phosphorylation of eNOS by Akt leads to enhanced enzymatic activity of eNOS that may contribute to TRAIL resistance of LNCaP cells. To test this hypothesis, we established a stable cell line of PC-3 cells overexpressing eNOS(S1179D), an eNOS mutant bearing a mutation at Ser1179 that possesses a higher enzymatic activity. As shown in Figure 3, the stable line #8 expressing eNOS was partially resistant to TRAIL comparing to the control PC-3 line. This result suggested that high level of active Akt and eNOS in LNCaP cells may be molecular determinants for its TRAIL resistance. Further characterization of these eNOS stable cell lines is underway.

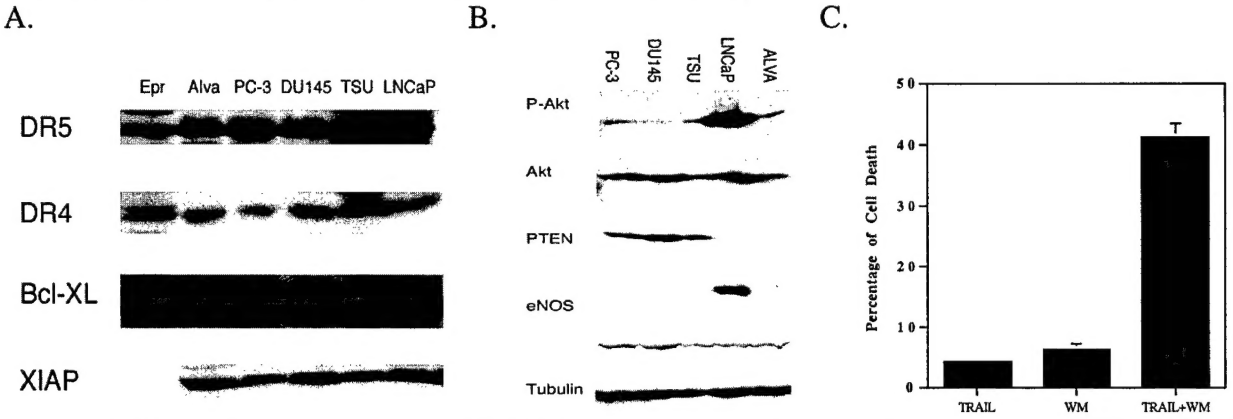

Figure 2. Molecular determinants of TRAIL sensitivity. A. Survey of components of TRAIL signaling pathway and its regulator. Lysates of five PC cell lines (ALVA-31, PC-3, DU145, TSU-Pr1 and LNCaP) and normal prostate epithelial cell (Epr) were subjected to immunoblotting using corresponding antibodies. B. LNCaP cells contain high level of active Akt and eNOS. Cell lysates were immunoblotted with specific antibody to phosphorylated Akt, Akt, PTEN, eNOS and tubulin. C. Wortammanin (WM) sensitized LNCaP cells to TRAIL. LNCaP cells were treated with either TRAIL alone (200 ng/ml), or wortamannin alone (100 nM), or the combination of both, for 24 hours. The percentage of cell death was evaluated by MTT assay.

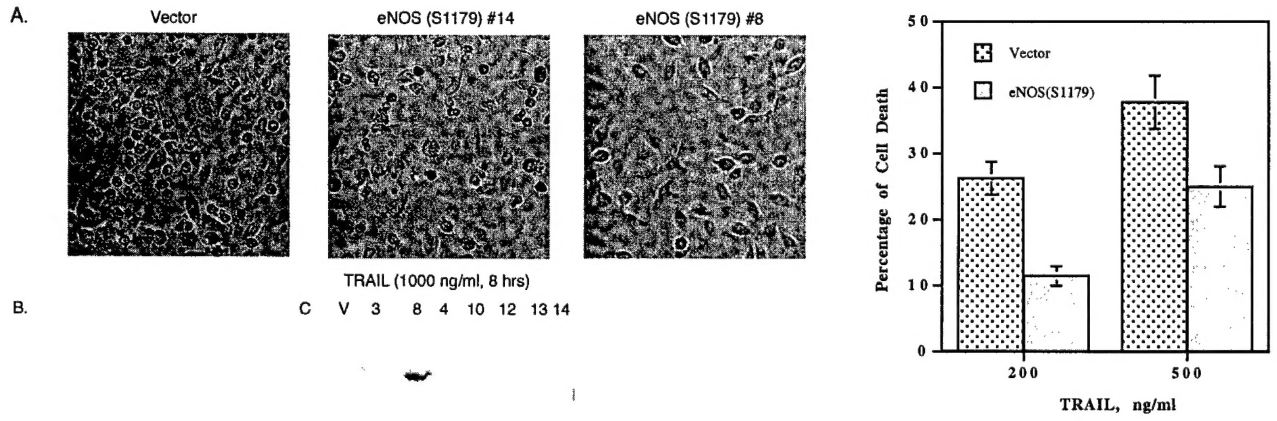

Figure 3. Overexpression of eNOS partially inhibits TRAIL-induced apoptosis of PC-3. A. Light microscopy of PC-3 stable cell lines treated with TRAIL. Three stable cell lines of PC-3 (vector control, #14 as an eNOS negative cell line, and #8 as an eNOS postive cell line) were treated with TRAIL (1000 ng/ml) for 8 hours. Round and shrunk cells were apoptotic cells. B. Expression level of eNOS(S1179D) in various stable cell lines of PC-3 cells. Cell lysates were immunoblotted with eNOS antibody. C. Inhibition of TRAIL-induced apoptosis of PC-3 cells by overexpression of eNOS(S1179D). The percentage of cell death was measured by MTT assay.

4) A novel caspase-8 inhibitor?

Although our study suggested that eNOS may play a regulatory role in TRAIL signaling pathway, overexpression of eNOS only partially inhibits TRAIL-induced apoptosis of PC-3 cells, indicating that other anti-apoptotic factors may exist. As shown in Figure 4, when LNCaP cells were treated with TRAIL in the absence of cycloheximide, a minimal cell death was detected (<5%). Interestingly, a large portion of caspase-8 was processed to its active form while its downstream targets BID and caspase-7 were not cleaved and activated. In contrast, in the presence of cycloheximide, massive cells underwent apoptosis, whereas caspase-7 and -8 were activated in proportion to the percentage of cell death. One possible explanation is that a novel inhibitor for active caspase-8 may exist. Alternatively, the active caspase-8 may not access to its downstream targets. Further study will address these different possibilities.

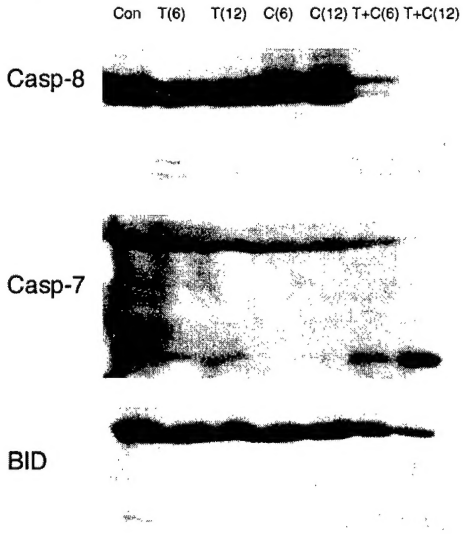

Figure 4. The cleavage of caspase-8 substrate but not activation of caspase-8 was inhibited in LNCaP cells treated with TRAIL. LNCaP cells were treated with either TRAIL alone (T) or cycloheximide (C) or the combination of both (T+C) for various periods of time (6 hours and 12 hours). Cell lysates were subjected to SDS-PAGE and immunoblotting with specific antibodies as indicated.

KEY RESEARCH ACCOMPLISHMENTS:

- We found that most of prostate cancer cells were sensitive to TRAIL treatment while normal prostate epithelial cells were resistant.
- We found that high level of constitutively active pro-survival protein kinase Akt existed in TRAIL-resistant LNCaP cells. Inhibition of PI 3-kinase sensitized LNCaP cells to TRAIL.
- We found that the elevated eNOS activity by Akt phosphorylation may contribute to Akt-mediated TRAIL resistance in LNCaP cells.
- Our preliminary result indicated that other anti-apoptotic mechanisms may exist in LNCaP cells.

REPORTABLE OUTCOMES:

Manuscript is in preparation.

CONCLUSIONS:

Our in vitro data suggest that TRAIL is effective to induce most of prostate cancer cells to undergo apoptosis but not toxic to normal prostate epithelial cells. This result lay a solid foundation for further investigation of TRAIL's effect on prostate cancer cells in vivo. Elucidation of TRAIL signaling pathway and its regulation help us to understand the molecular mechanism of TRAIL resistance. Overcome of the anti-apoptotic mechanism, such as Akt's anti-apoptotic activity, in TRAIL-resistant cells by specific inhibitors will be the key for TRAIL-based therapy.

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